

Inhibition of Smooth Muscle Proliferation by Urea-Based Alkanoic Acids via Peroxisome Proliferator-Activated Receptor α -Dependent Repression of Cyclin D1

Valerie Y. Ng, Christophe Morisseau, John R. Falck, Bruce D. Hammock, Deanna L. Kroetz

Objective—Proliferation of smooth muscle cells is implicated in cardiovascular complications. Previously, a urea-based soluble epoxide hydrolase inhibitor was shown to attenuate smooth muscle cell proliferation. We examined the possibility that urea-based alkanoic acids activate the nuclear receptor peroxisome proliferator-activated receptor α (PPAR α) and the role of PPAR α in smooth muscle cell proliferation.

Methods and Results—Alkanoic acids transactivated PPAR α , induced binding of PPAR α to its response element, and significantly induced the expression of PPAR α -responsive genes, showing their function as PPAR α agonists. Furthermore, the alkanoic acids attenuated platelet-derived growth factor–induced smooth muscle cell proliferation via repression of cyclin D1 expression. Using small interfering RNA to decrease endogenous PPAR α expression, it was determined that PPAR α was partially involved in the cyclin D1 repression. The antiproliferative effects of alkanoic acids may also be attributed to their inhibitory effects on soluble epoxide hydrolase, because epoxyeicosatrienoic acids alone inhibited smooth muscle cell proliferation.

Conclusions—These results show that attenuation of smooth muscle cell proliferation by urea-based alkanoic acids is mediated, in part, by the activation of PPAR α . These acids may be useful for designing therapeutics to treat diseases characterized by excessive smooth muscle cell proliferation. (*Arterioscler Thromb Vasc Biol.* 2006;26:2462-2468.)

Key Words: soluble epoxide hydrolase ■ epoxyeicosatrienoic acids ■ PPAR α ■ smooth muscle cells ■ proliferation

Smooth muscle cell (SMC) proliferation is a critical event in atherosclerosis¹ and in restenosis following interventional procedures.² After injury to the vasculature, a vasculoproliferative cascade is initiated that includes the recruitment and proliferation of SMCs,³ which can eventually lead to occlusive lesions that result in myocardial ischemia.⁴ Much effort has been made to inhibit SMC proliferation using pharmacological and genetic approaches that interfere with cell cycle regulators such as cyclins⁵ and cyclin-dependent kinases (CDKs).⁶

Cyclins and CDKs are part of the regulatory machinery controlling cell cycle progression. The D and E cyclins and their associated kinases are viewed as essential for entry into and progression through the G₁ phase of a cell cycle.⁷ Overexpression of the D cyclins can shorten G₁, implying that they are critical for cell cycle progression through this phase.⁸ In leukemic and breast cancer cells, xenobiotics such as clofibrate and troglitazone inhibit cyclin D expression, resulting in cell cycle arrest.^{9,10} These compounds are ligands for the peroxisome proliferator-activated receptor (PPAR) (NR1C) family of nuclear receptors.

PPARs are ligand-activated nuclear receptors of which there are 3 isoforms (α , γ , and δ). Activators of PPAR α include polyunsaturated fatty acids and fibrate drugs. PPAR γ ligands include the prostaglandin (PG) D₂ derivative 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15- Δ PGJ₂), oxidized linoleic acid, and the antidiabetic thiazolidinediones such as troglitazone and rosiglitazone.¹¹ All 3 PPAR isoforms are expressed in vascular smooth muscle and endothelial cells, and recent studies have elucidated the importance of these receptors in atherogenesis.¹² On ligand activation, PPAR heterodimerizes with the retinoid X receptor (RXR), and they subsequently bind to the peroxisome proliferator response element (PPRE). By recruiting large complexes of coactivators, target gene transcription is then initiated. PPARs can also repress gene expression by interfering with other signaling pathways such as the nuclear factor κ B and activator protein-1 pathways.^{13,14}

Eicosanoids derived from the cytochrome P450–catalyzed metabolism of arachidonic acid include the regio- and stereoisomeric epoxyeicosatrienoic acids (EETs), which have potent vasoactive and antiinflammatory effects in smooth muscle and endothelial cells.¹⁵ The soluble epoxide hydrolase

Original received July 18, 2006; final version accepted August 4, 2006.

From the Department of Biopharmaceutical Sciences (V.Y.N., D.L.K.), the Center for Human Genetics (D.L.K.), and the Liver Center (D.L.K.), University of California, San Francisco; Department of Entomology and the Cancer Research Center (C.M., B.D.H.), University of California, Davis; and Department of Biochemistry (J.R.F.), University of Texas Southwestern Medical Center, Dallas.

Correspondence to Deanna L. Kroetz, PhD, Department of Biopharmaceutical Sciences, University of California San Francisco, 1550 4th St, Box 2911, San Francisco, CA 94143-2911. E-mail deanna.kroetz@ucsf.edu

© 2006 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at <http://www.atvbaha.org>

DOI: 10.1161/01.ATV.0000242013.29441.81

(sEH) enzyme hydrates EETs into their corresponding dihydroxyeicosatrienoic acids (DHETs) and is thus critical in controlling the biological effects of EETs. Interestingly, sEH is strongly induced by PPAR α ligands such as clofibrate.¹⁶ In a recent study, 1-cyclohexyl-3-dodecyl urea (CDU), a urea-based sEH inhibitor, decreased platelet-derived growth factor (PDGF)-induced SMC proliferation by inhibiting cyclin D1 expression.¹⁷ This study suggested an increase in intracellular EET concentration caused by inhibition of sEH may be responsible for the decrease in SMC proliferation. However, other studies have indicated that EETs are mitogenic in SMCs.¹⁸ Thus the ability of CDU to inhibit SMC proliferation may be independent of its effects on sEH. The effect of CDU may also be context dependent, varying with species, tissue, and physiological state.

In this report, we show that inhibitors of sEH, the urea-based alkanolic acids, activate PPAR α and in turn attenuate PDGF-induced SMC proliferation by repressing cyclin D1 expression. To unambiguously determine whether the decreased cyclin D1 expression is mediated by PPAR α , endogenous expression of PPAR α in SMCs was decreased using small interfering RNA (siRNA). Results indicate that PPAR α is at least partially responsible for the observed attenuation of SMC proliferation by urea-based alkanolic acids.

Methods

Materials and Cell Culture

The synthesis of all urea-based acids has been described in detail elsewhere.¹⁹ The compounds used in this study were cyclohexyl butanoic acid urea (CUBA), cyclohexyl heptanoic acid urea (CUHpA), cyclohexyl octanoic acid urea (CUOA), cyclohexyl undecanoic acid urea (CUUA), cyclohexyl dodecanoic acid urea (CUDA), adamantyl dodecanoic acid urea (AUDA), and cyclohexyl dodecyl urea (CDU). The cyclin D1 and β -actin antibodies were purchased from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. Wy 14,643 and PDGF-BB were purchased from Biomol and Upstate Biotechnology, respectively. Radioactive compounds were obtained from Perkin Elmer. Human aortic and coronary artery SMCs were purchased from Cambrex and maintained in SmGM-2 medium. All experiments using SMCs were carried out at passages 4 to 9.

HepG2 and SMC Transfection

HepG2 cells were transfected in 24-well plates with 1 ng Gal4-hPPAR α , 100 ng UAS $_4$ -LUC (both kindly provided by Dr Thomas Scanlan at the University of California, San Francisco), and 20 ng of pCMV- β gal using Lipofectamine PLUS reagents (Invitrogen) according to the instructions of the manufacturer. Twenty-four hours after transfection, cells were treated with test compounds dissolved in DMSO (<0.1%) for an additional 24 hours. Luciferase activity is normalized to β -galactosidase activity, and fold activation is calculated relative to the DMSO control. Each experiment was carried out in triplicate and repeated 3 times. For the detection of PPAR α responsive genes, cells were transfected with 1 ng of pCMX-mPPAR α (kindly provided by Dr Ronald Evans at the Salk Institute, La Jolla, Calif).

Aortic SMCs were grown to 50% to 60% confluence and transfected with PPAR α (no. 5439) or negative control siRNA no. 1 using siPORT *Amine* (all from Ambion) according to the instructions of the manufacturer. For detection of cyclin D1 expression, transfected cells were incubated in quiescence medium 24 hours after transfection for 1 day. Cells were then exposed to growth medium with or without the test compounds for the indicated times.

Gel Shift Assays

pCMX-mPPAR α and pRS-hRXR α were translated using the TNT reticulocyte in vitro translation system (Promega). Plasmids were kindly provided by Dr Ronald Evans (Salk Institute). Gel shift assays were carried out as described previously.²⁰ The sequence for the consensus PPRE is 5'-CAA AAC TAG GTC AAA GGT CA-3'; for the mutant PPRE, 5'-CAA AAG TAG CAC AAA GCA CA-3'.

[³H]-Thymidine Incorporation, Real-Time Quantitative Polymerase Chain Reaction, and Western Immunoblotting

Incorporation of [³H]-thymidine into SMCs was assessed as described.¹⁷ Total RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed using M-MLV reverse transcriptase (Promega). The 18S primers and probe were provided by the University of California San Francisco Cancer Center. All other primers and probes were purchased from Applied Biosystems. Relative expression of specific transcripts was calculated by the following formula: relative expression = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (\Delta Ct_{\text{target}} - \Delta Ct_{18S})_{\text{treated}} - (\Delta Ct_{\text{target}} - \Delta Ct_{18S})_{\text{control}}$.²¹ For Western immunoblotting, cells were lysed with a 1% Igepal lysis buffer and proteins were separated on Criterion 12.5% Tris-HCl gels (Bio-Rad) and transferred onto nitrocellulose membranes. Membranes were incubated with a 1:1000 dilution of primary antibody followed by incubation with horseradish peroxidase-conjugated secondary antibody.

Statistics

Statistical significance of differences between values was evaluated by an unpaired Student's *t* test. Significance was set at a probability value of <0.05.

Results

Urea-Based Alkanoic Acids Activate PPAR α and Induce Formation of a PPAR α /PPRE Complex

An adamantyl and a series of cyclohexyl substituted urea-based alkanolic acids were tested for their ability to activate PPAR α . The structures of these acids are shown in supplemental Figure I of the online data supplement, available at <http://atvb.ahajournals.org>. In transactivation assays, HepG2 cells were transfected with the DNA-binding domain of the Gal4 transcription factor fused to the ligand-binding domain of the hPPAR α receptor and tested for their ability, in the presence of the alkanolic acids, CDU, or the PPAR α -positive control Wy 14,643, to transactivate the Gal4 response element UAS tagged to a luciferase reporter gene (UAS $_4$ -LUC). All of the alkanolic acids and CDU significantly activated PPAR α compared with the DMSO vehicle control (Figure 1A). PPAR α activation increased as the carbon chain length in the cyclohexyl-based acids was lengthened. A maximum activation was observed with CUUA and CUDA. The adamantyl urea-based acid AUDA also strongly activated PPAR α more than 20-fold over control. As expected, activation required PPAR α transfection. A steep dose dependence for PPAR α activation was observed between 10 and 100 $\mu\text{mol/L}$ CUUA and AUDA, with saturation evident at 50 $\mu\text{mol/L}$ (supplemental Figure II). The Gal4-thyroid receptor β was not transactivated by CUUA or AUDA, showing that activation of nuclear receptors is specific for PPAR α (data not shown).

Using electrophoretic mobility-shift assays (EMSA), it was next examined whether these alkanolic acids could induce the formation of a PPAR α -PPRE complex. In vitro-translated PPAR α and RXR α were tested for their ability to heterodimerize in the presence of Wy 14,643 or the alkanolic

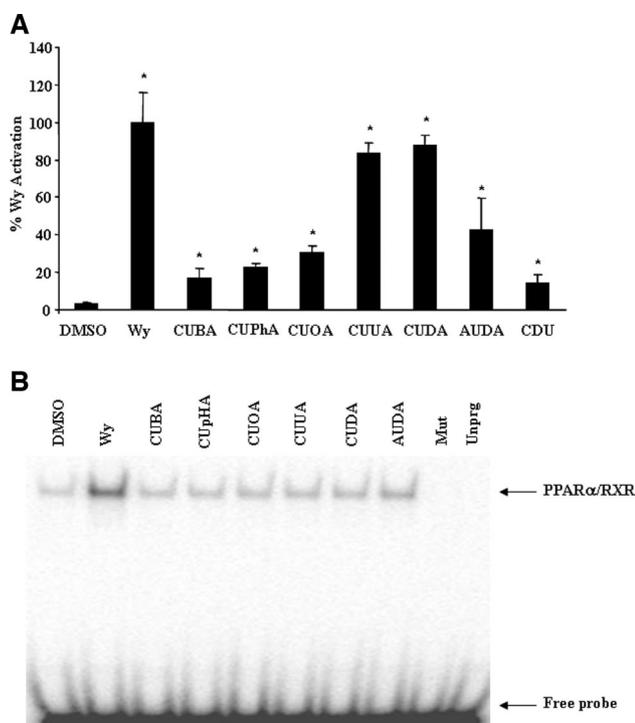


Figure 1. Urea-based alkanolic acids activate and bind to PPAR α . A, Transactivation of PPAR α after 24 hours of incubation with 50 μ mol/L alkanolic acid, CDU, or Wy 14,643. Luciferase values were normalized to β -galactosidase, and fold activation was calculated as increase over the DMSO control with Wy 14,643 activation set at 100%. Values shown are the mean \pm SD of a representative experiment performed in triplicate. * P <0.05 compared with DMSO. B, EMSAs with PPAR α and RXR α were performed by adding 50 μ mol/L Wy 14,643 or alkanolic acid ureas. A mutant PPRE (mut) and unprogrammed reticulocyte (unprg) were used as controls.

acids and bind to the PPRE. Addition of Wy 14,643 or the alkanolic acids resulted in the detection of a PPRE-protein complex (Figure 1B). PPAR α and RXR α were also able to heterodimerize and bind the PPRE in the absence of ligand; however, densitometric analysis revealed that the extent of this basal binding was significantly less than the binding observed with Wy 14,643 or all of the alkanolic acids except CUBA and CUHpA. The negative controls, a mutated response element and unprogrammed lysates, did not result in heterodimer formation and DNA binding. The DNA-protein complex was supershifted by PPAR α - and RXR α -specific antibodies, and unlabeled PPRE competed for PPAR α /RXR α binding to the PPRE (supplemental Figure III).

Urea-Based Alkanolic Acids Induce Expression of PPAR α -Responsive Genes

To determine whether these PPAR α activators could affect known PPAR α -responsive genes, acyl-coenzyme A (CoA) synthetase (ACS), carnitine palmitoyl transferase 1A (CPT1A), and acyl-CoA oxidase (ACOX) mRNA expression were determined in hepatocytes, and cyclooxygenase-2 (COX-2) expression was assessed in human aortic SMCs using TaqMan quantitative polymerase chain reaction (PCR) (Figure 2). Treatment of PPAR α -transfected HepG2 cells with Wy 14,643, CUUA, or AUDA resulted in the induction

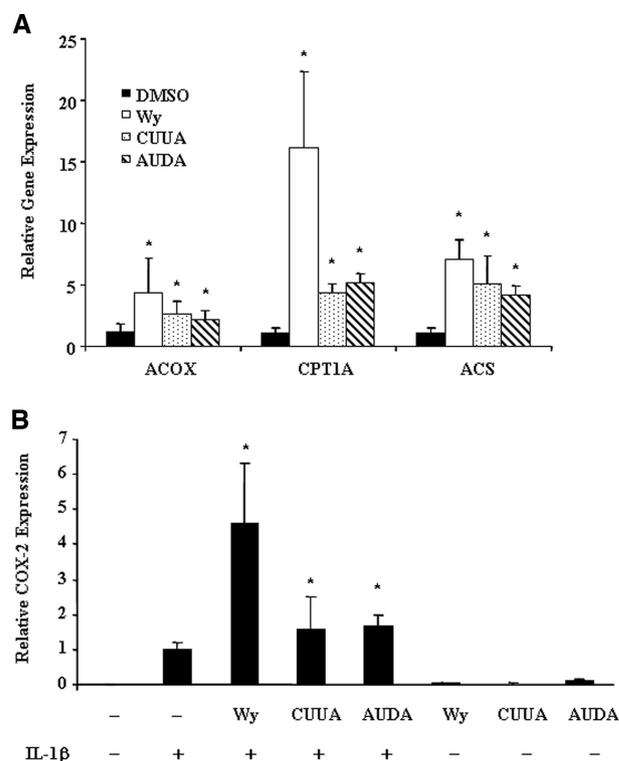


Figure 2. CUUA and AUDA induce PPAR α -responsive genes. A, HepG2 cells were treated for 24 hours with 50 μ mol/L Wy 14,643, CUUA, or AUDA, and ACS, CPT1A, and ACOX expression were determined using real-time quantitative PCR. B, Human SMCs were treated as in A but for 6 hours with or without 1 ng/mL IL-1 β , and COX-2 expression was determined. Target gene expression was normalized to 18S expression. The values shown are the mean \pm SD of a representative experiment performed in triplicate. * P <0.05 between control and treated cells.

of all 3 hepatic PPAR α -responsive genes (Figure 2A). ACS expression was upregulated 7-fold, CPT1A 16-fold, and ACOX1 5-fold by Wy 14,643. CUUA and AUDA increased ACS expression 4- to 5-fold. Similarly, CPT1A and ACOX expression were induced 2- to 5-fold by CUUA and AUDA. The expression of the PPAR α -responsive gene COX-2 in SMCs was determined in the absence or presence of interleukin (IL)-1 β (Figure 2B). Wy 14,643 potentiated IL-1 β -induced COX-2 expression by 4-fold, whereas CUUA and AUDA both increased COX-2 expression by approximately 2-fold.

Urea-Based Alkanolic Acids Inhibit Human SMC Proliferation

Human aortic SMCs were quiesced for 24 hours, then incubated in medium containing PDGF-BB with or without the PPAR α activators Wy 14,643, CUUA, or AUDA. As expected, PDGF resulted in a marked increase in SMC proliferation (Figure 3). Wy 14,643, CUUA, and AUDA, but not CDU, significantly attenuated PDGF-induced proliferation (Figure 3A). In dose-dependence studies, a significant decrease in proliferation relative to PDGF alone was evident starting at 1 μ mol/L CUUA or AUDA (Figure 3B). Interestingly, a sharp decrease in proliferation was observed when human aortic SMCs were treated with 20 μ mol/L AUDA. A

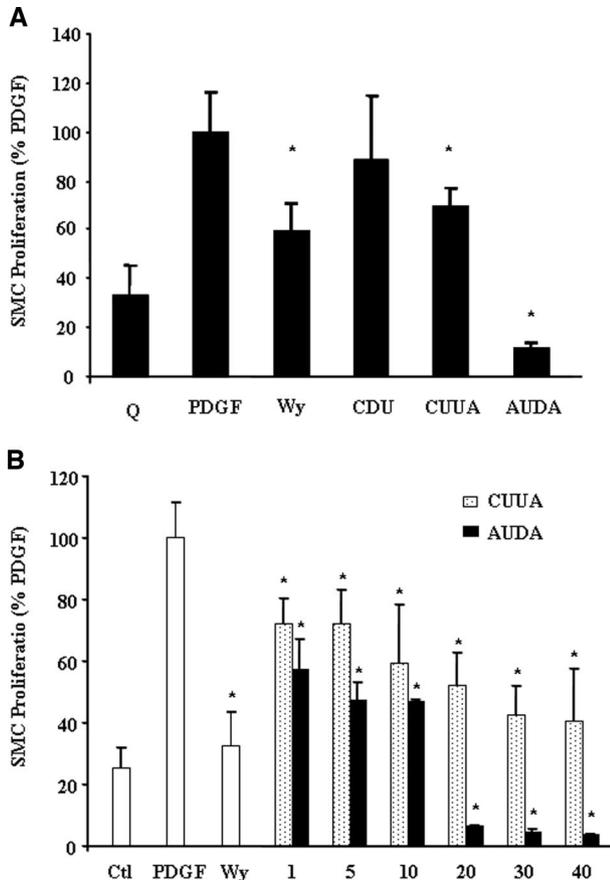


Figure 3. CUUA and AUDA decrease proliferation in human SMCs. [³H]-Thymidine incorporation was measured in aortic SMCs incubated for 24 hours in quiescent (Q) or PDGF medium containing 50 μmol/L Wy 14,643, CDU, CUUA, or AUDA (A) or 1 to 40 μmol/L CUUA or AUDA (B). The values shown are expressed as the mean ± SD of a representative experiment performed in triplicate. **P* < 0.05 over PDGF positive control.

similar decrease was observed when coronary artery SMCs were treated with 20 μmol/L AUDA (data not shown). To show that decreased SMC proliferation is not attributed to increased cellular toxicity, SMCs were subjected to proliferation conditions and fluorescently labeled with a viability stain, 7-AAD (supplemental Figure IV). Cellular toxicity was not evident in any of the treatments and thus cannot account for the attenuation of SMC proliferation

Urea-Based Alkanoic Acids Decrease Cyclin D1 Expression, an Effect That Requires, in Part, the Expression of PPAR α

To understand the mechanism whereby CUUA and AUDA induce cell cycle arrest, cyclin D1 RNA and protein levels were examined (Figure 4). Cyclin D1 RNA expression was induced 6 to 12 hours after incubation in PDGF medium (Figure 4A). With the addition of Wy 14,643 and AUDA, cyclin D1 levels were significantly decreased at 6 to 24 hours, and CUUA attenuated cyclin D1 RNA expression at 12 hours compared with PDGF treatment alone. Increased cyclin D1 protein levels are detected already at 6 hours following PDGF treatment, consistent with its early expression in the cell cycle (Figure 4B). Addition of Wy 14,643, CUUA, and AUDA

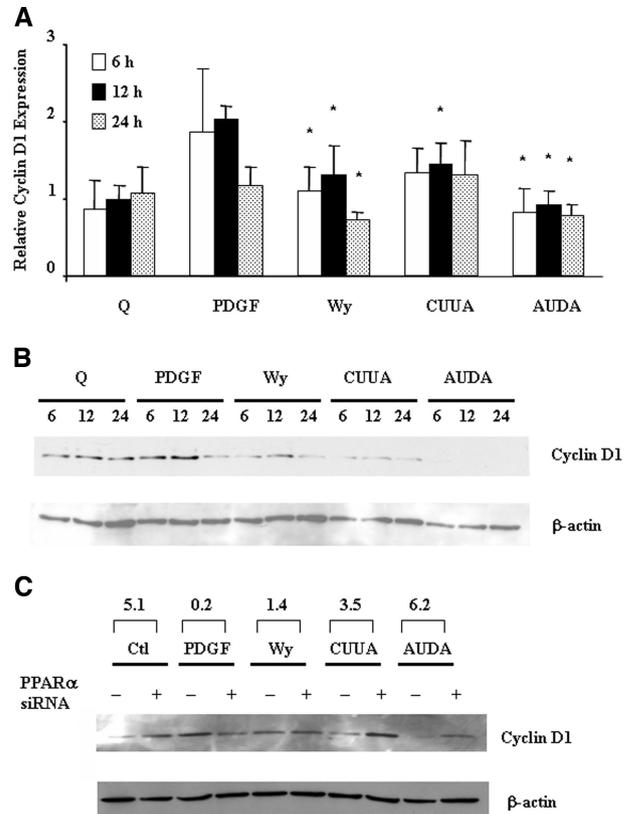


Figure 4. CUUA and AUDA treatment attenuate cyclin D1 expression and decreased PPAR α levels partially alleviate the attenuation. Cyclin D1 RNA (A) and protein (B) levels in human SMCs were measured after treatment of SMCs in PDGF medium containing 50 μmol/L Wy 14,643, 30 μmol/L CUUA, or 30 μmol/L AUDA. C, SMCs were transfected for 24 hours with PPAR α -specific siRNA or negative control siRNA and treated as described in A. Cyclin D1 mRNA expression was quantified by real-time quantitative PCR and normalized to 18S. The values shown are the mean ± SD of a representative experiment performed in triplicate. **P* < 0.05 over PDGF-positive control at a given time point. Cyclin D1 protein expression was determined by Western immunoblotting using β -actin as a loading control. The fold difference in cyclin D1 expression between PPAR α and empty vector siRNA-transfected cells is noted in C for each tested compound.

attenuated PDGF-induced cyclin D1 protein expression from 6 to 24 hours, with AUDA almost completely ablating cyclin D1 expression at all time points examined. To determine whether inhibition of cyclin D1 is attributed to CUUA/AUDA activation of PPAR α , PPAR α expression was silenced using siRNA in SMCs (Figure 4C and supplemental Figure V). Transfection with the negative control siRNA had no effect on PPAR α transcript or protein expression. In SMCs transfected with negative control siRNA, cyclin D1 protein expression was induced with the addition of PDGF medium and attenuated in the presence of Wy 14,643, CUUA, and AUDA. These results indicated that transfection of SMCs with the control siRNA did not interfere with the normal response of cyclin D1 to PPAR α activators. Interestingly, in PPAR α siRNA-transfected SMCs, PDGF failed to induce cyclin D1, whose level was comparable to that in growth-arrested cells. Because Wy 14,643 is a known agonist of PPAR α , silencing of PPAR α should dampen the attenua-

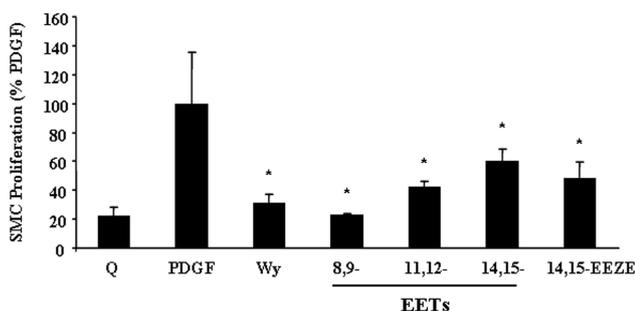


Figure 5. EETs and 14,15-EEZE inhibit PDGF-induced proliferation. [3 H]-Thymidine incorporation was measured as described in the legend of Figure 3, except that cells were treated with 10 μ M EETs or 14,15-EEZE for 24 hours. The values shown are the mean \pm SD of a representative experiment performed in triplicate. * P <0.05 over PDGF positive control.

tion of cyclin D1 expression that was observed in control-transfected cells. Consistent with this reasoning, SMCs transfected with PPAR α siRNA and treated with PDGF medium containing Wy 14,643 resulted in a 1.4-fold increase in cyclin D1 expression. Furthermore, the repressive effects of CUUA and AUDA were partially mitigated by knocking down PPAR α . In CUUA-treated cells, cyclin D1 expression increased 3.5-fold relative to control-transfected cells, and a striking 6.2-fold increase was observed with AUDA treatment. These results show that PPAR α plays a role in mediating the inhibitory effects of CUUA and AUDA on cyclin D1 expression.

EETs and 14,15-EEZE Inhibit PDGF-Induced SMC Proliferation

To determine whether the antiproliferative effects of CUUA and AUDA can be attributed to their inhibition of sEH and subsequent elevation of cellular EET levels, proliferation assays were performed with various regioisomeric EETs (Figure 5). It was first determined that sEH expression was detectable in these cells (supplemental Figure VI). EETs were indeed effective against SMC proliferation, with 8,9-, 11,12-, and 14,15-EET decreasing PDGF-induced proliferation by 77%, 57%, and 40%, respectively. The putative EET receptor antagonist 14,15-epoxyeicosa-5(Z)-enoic acid (EEZE) was also used to attenuate the EET-dependent effects. Interestingly however, 14,15-EEZE itself significantly inhibited SMC proliferation (Figure 5).

Discussion

Urea-based alkanolic acids are potent inhibitors of sEH with nanomolar K_i values.¹⁹ The studies described within demonstrate that the urea-based alkanolic acids CUUA and AUDA functionally activated PPAR α and induced the expression of PPAR α -responsive genes in hepatocytes and SMCs. Because the concentration required for PPAR α activation is in the micromolar range, the effects of sEH inhibition may be distinct from that of PPAR α activation. CUUA and AUDA also attenuated PDGF-induced SMC proliferation and repressed cyclin D1 expression, an action mediated partially by PPAR α . However, we cannot rule out the possibility that increased EET levels resulting from sEH inhibition by CUUA and AUDA contributes to the antiproliferative effects of these compounds. CUUA, AUDA, and related compounds may

represent novel structural leads for the synthesis of more potent PPAR α ligands for therapeutic use.

Cyclohexyl alkanolic acid and adamantyl-dodecanoic acid ureas were tested as putative PPAR α activators. Functional assays showed increasing activity with increasing alkyl chain length, with a maximum effect observed with the 11 and 12 carbon analogs CUUA and CUDA. The crystal structure of AZ 242 bound to the ligand-binding domain of human PPAR α shows extensive hydrophobic interactions between its central ring systems and the receptor.²² The fact that shorter chain alkanolic ureas are less effective as PPAR α activators may be attributable to decreased hydrophobic interactions. CUDA and AUDA differ only in the substitution of the cyclohexyl for an adamantyl group, and AUDA is a slightly weaker PPAR α activator. The bulkier adamantyl moiety might restrict accessibility to the ligand binding site; however, this effect is not limiting because AUDA potently transactivates PPAR α .

During the preparation of this report, a similar study was published that reported the activation of PPAR α by CUDA and AUDA.²³ Reminiscent of our findings, the authors showed that chain-shortened metabolic products of CUDA and AUDA were progressively less potent activators of PPAR α . In addition, the PPAR α agonist GW7647 reduced DHET production by 30% to 50%, suggesting that PPAR α agonists and alkanolic acid ureas can be rationally optimized to perform both biological functions.

Via PPAR α -mediated mechanisms, peroxisome proliferators induce the expression of numerous genes involved in fatty acid metabolism and transport in the liver, including ACS, CPT1A, and ACOX1.^{24–26} The novel PPAR α activators, CUUA and AUDA, are inducers of ACS, CPT1A, and ACOX1, critical enzymes that facilitate the uptake and entry of fatty acids into the mitochondria for catabolism by β -oxidation.²⁷ It is therefore possible that treatment with alkanolic acid urea-based sEH inhibitors may influence lipid homeostasis.

In vascular SMCs, PPAR α -responsive genes are expressed in response to numerous inflammatory stimuli.¹¹ One such gene is COX-2, which has a PPRE in its promoter region.²⁸ In this study, we show that in human aortic SMCs, CUUA and AUDA potentiate IL-1 β -induced expression of COX-2. The consequences of increased COX-2 expression in SMCs in response to sEH inhibitors are not yet clear. Studies have reported that upregulation of COX-2 expression and its metabolites can result in both increased and decreased SMC proliferation.^{29,30} Interestingly, COX-2 can have inflammatory properties in venules and act as an antiinflammatory mediator in SMCs of large capacity vessels.³⁰ An understanding of the role of COX-2 metabolites in the vascular response to inflammatory stimuli will be necessary for evaluating the use of sEH inhibitors for the treatment of vascular inflammation.

Furthermore, CUUA and AUDA attenuated PDGF-induced SMC proliferation. Another urea-based compound, CDU, was previously shown to inhibit SMC proliferation¹⁷; however, we have not been able to consistently demonstrate its antiproliferative effects. This discrepancy is likely attributable to the use of primary cells from different donors. Urea-based alkanolic acids inhibit sEH at nanomolar concentrations, whereas their antiproliferative effect was only observed at micromolar concentrations, suggesting that the

inhibitors may signal via a separate pathway to decrease SMC proliferation. AUDA decreased proliferation by 50% at a concentration of 5 $\mu\text{mol/L}$, whereas 5 $\mu\text{mol/L}$ CUUA only moderately inhibited SMC proliferation. The fact that CUUA and AUDA have relatively comparable effects on PPAR α activation, yet CUUA is not as potent at inhibiting SMC proliferation as AUDA, suggests that the antiproliferative effect of these compounds may not be solely attributable to PPAR α activation. A steep decrease in proliferation was observed between 10 $\mu\text{mol/L}$ and 20 $\mu\text{mol/L}$ AUDA in 2 different types of SMCs, and toxicity could not account for the sudden proliferative decrease. Possibly, the phenomenon is associated with the ability of AUDA to form aggregates (B. Feng, V.Y. Ng, D.L. Kroetz, B.K. Shoichet, unpublished data, 2005). Small molecules may inhibit enzymes nonspecifically by the formation of aggregates at micromolar concentrations.³¹ At 20 $\mu\text{mol/L}$ AUDA, aggregates form readily, whereas minimal aggregation was observed with the same concentration of CUUA. Surprisingly, AUDA is 2 times more soluble in water than CUUA; therefore, their aggregatory properties may not be related to their solubility.¹⁹ It may be possible that AUDA, by forming aggregates, indirectly inhibits cyclin D1 expression. Unfortunately it was not possible to test this hypothesis because the required disruption of aggregates using Triton-X is detrimental to cellular integrity. Also the adamantane group is more rapidly metabolized by hydroxylation in some cases than is the cyclohexyl. Thus differential metabolism is a possible explanation, but investigation is beyond the scope of this study.

Inhibition of cyclin D1 RNA and protein was observed when PDGF-stimulated SMCs were incubated with AUDA. AUDA completely abolished cyclin D1 protein expression, indicating SMCs were G₁ growth arrested. Despite the complete repression of cyclin D1 immunoreactive protein, its transcripts were readily detectable. The discrepancy could be attributable to the high sensitivity of TaqMan real-time PCR or that expression of cyclin D1 is regulated by both transcriptional and nontranscriptional mechanisms. Interestingly, CUUA inhibited cyclin D1 protein expression at 6 and 12 hours yet had minimal effects on cyclin D1 RNA. These results would suggest that CUUA does not regulate cyclin D1 at the transcriptional level. Nontranscriptional mechanisms for nuclear receptor signaling have been reported. Estrogen receptor regulates nitric oxide release by physically interacting with the regulatory subunit of the lipid kinase phosphatidylinositol 3-kinase³² and mediates estrogen-dependent transport of ribonucleoprotein from the nucleus to the cytoplasm.³³ It is therefore plausible that CUUA and AUDA exert their effects on cyclin D1 via nontranscriptional mechanisms.

An alternative explanation would suggest that CUUA and AUDA inhibit SMC proliferation independent of PPAR α ; however, by reducing its expression, the role of PPAR α was evident. In the absence of normal levels of PPAR α , repression of cyclin D1 by alkanolic acids was greatly reduced. Interestingly, when PPAR α expression is reduced, PDGF failed to induce expression of cyclin D1, indicating that PPAR α may be a downstream member of the PDGF signaling pathway. PPAR ligands have been implicated in the progression of the cell cycle. In rat aortic SMCs, a partial PPAR γ agonist attenuated the

induction of G₁ cyclins by PDGF, and in human leukemic cells, clofibrate arrested cells in G₀/G₁ by decreasing cyclin D2 and c-myc expression.^{10,34} Without complete knockdown of PPAR α , it is not possible to conclude that CUUA and AUDA repress cyclin D1 solely by activating PPAR α .

Urea-based inhibitors have been widely used to block the conversion of EETs into DHETs and to examine the role of EETs in numerous biological functions. To date, effects associated with sEH inhibition have been attributed to the increased levels of EETs or more generally the increase in lipid epoxide to diol ratios, including the reduction of blood pressure and protection against kidney failure in hypertensive rats.^{35,36} In this report, EETs alone inhibited SMC proliferation. We have also demonstrated that EETs are potent activators of PPAR α (V.Y. Ng, L.M. Reddy, J.R. Falck, D.L. Kroetz, unpublished data); therefore, it is possible that the PPAR α -activating and sEH inhibitory effects of the alkanolic acids act in concert to culminate in their anti-proliferative properties. PPAR α activation and sEH inhibition/EET overexpression display overlapping biological effects, such as their antiinflammatory properties in vascular cells,^{13,37} and protective effects during hypoxic and ischemic conditions.^{38–41} Reexamination of PPAR α functions in the studies that involved the use of sEH inhibitors or overexpression of cytochrome P450 epoxygenases will provide additional insight into its role in these disease models.

Interestingly, the putative EET antagonist 14,15-EEZE also inhibited SMC proliferation. This compound has been shown to both inhibit and enhance relaxation of arteries^{42,43}; thus its actions have not been clearly defined. To date, the activity of 14,15-EEZE in SMCs has not been studied. It is possible that 14,15-EEZE, being a structural analog of EETs, acts as a sEH substrate such that sEH activity is inhibited and EET levels are increased.

In summary, the present study identified a novel class of PPAR α activators, urea-based alkanolic acids, which are also inhibitors of sEH. CUUA and AUDA can induce fatty acid metabolism as well as inhibit SMC proliferation. Because excess SMC accumulation and proliferation is a hallmark of atherosclerosis, the use of these compounds for treatment of atherosclerosis is an intriguing possibility. In addition to possessing qualities of other PPAR α agonists, CUUA and AUDA have the added ability to inhibit sEH, thereby increasing vascular protective EET levels. The combined biological effects of these urea-based alkanolic acids may prove to be well suited for the treatment of vascular diseases such as hypertension and inflammation.

Acknowledgments

We acknowledge the help of Jason F. Kreisberg for assistance with fluorescence-activated cell sorter analysis and Brian Shoichet and Brian Feng for aggregation analysis of the sEH inhibitors.

Sources of Funding

This work was supported by grants from the National Institutes of Health (R01 HL53994 to D.L.K.; R37 ES02710, P42 ES04699, P30 ES05707, and R01 HL59699-06A1 to C.M. and B.D.H.; and R01 GM31278 to J.R.F.). V.Y.N was supported in part by predoctoral fellowships from the Pharmaceutical Research and Manufacturers of America Foundation and the American Foundation for Pharmaceutical Education.

Disclosures

D.L.K., B.D.H., and C.M. are authors of patents related to the use of sEH inhibitors for the treatment of hypertension and related cardiovascular disease.

References

- Ross R. The pathogenesis of atherosclerosis—an update. *N Engl J Med.* 1986;314:488–500.
- Virmani R, Farb A. Pathology of in-stent restenosis. *Curr Opin Lipidol.* 1999;10:499–506.
- Clowes AW, Reidy MA, Clowes MM. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest.* 1983;49:327–333.
- Sriram V, Patterson C. Cell cycle in vasculoproliferative diseases: potential interventions and routes of delivery. *Circulation.* 2001;103:2414–2419.
- Zhu NL, Wu L, Liu PX, Gordon EM, Anderson WF, Starnes VA, Hall FL. Downregulation of cyclin G1 expression by retrovirus-mediated antisense gene transfer inhibits vascular smooth muscle cell proliferation and neointima formation. *Circulation.* 1997;96:628–635.
- Morishita R, Gibbons GH, Ellison KE, Nakajima M, von der Leyen H, Zhang L, Kaneda Y, Ogihara T, Dzau VJ. Intimal hyperplasia after vascular injury is inhibited by antisense cdk 2 kinase oligonucleotides. *J Clin Invest.* 1994;93:1458–1464.
- Sherr CJ, Roberts JM. Living with or without cyclins and cyclin-dependent kinases. *Genes Dev.* 2004;18:2699–2711.
- Resnitzky D, Gossen M, Bujard H, Reed SI. Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol Cell Biol.* 1994;14:1669–1679.
- Yin F, Wakino S, Liu Z, Kim S, Hsueh WA, Collins AR, Van Herle AJ, Law RE. Troglitazone inhibits growth of MCF-7 breast carcinoma cells by targeting G1 cell cycle regulators. *Biochem Biophys Res Commun.* 2001;286:916–922.
- Laurora S, Pizzimenti S, Briatore F, Fraioli A, Maggio M, Reffo P, Ferretti C, Dianzani MU, Barrera G. Peroxisome proliferator-activated receptor ligands affect growth-related gene expression in human leukemic cells. *J Pharmacol Exp Ther.* 2003;305:932–942.
- Bishop-Bailey D. Peroxisome proliferator-activated receptors in the cardiovascular system. *Br J Pharmacol.* 2000;129:823–834.
- Marx N, Duez H, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors and atherogenesis: regulators of gene expression in vascular cells. *Circ Res.* 2004;94:1168–1178.
- Marx N, Sukhova GK, Collins T, Libby P, Plutzky J. PPAR α activators inhibit cytokine-induced vascular cell adhesion molecule-1 expression in human endothelial cells. *Circulation.* 1999;99:3125–3131.
- Irukayama-Tomobe Y, Miyachi T, Kasuya Y, Sakai S, Goto K, Yamaguchi I. Activation of peroxisome proliferator-activated receptor- α decreases endothelin-1-induced p38 mitogen-activated protein kinase activation in cardiomyocytes. *J Cardiovasc Pharmacol.* 2004;44:S358–S361.
- Roman RJ. P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev.* 2002;82:131–185.
- Hammock BD, Ota K. Differential induction of cytosolic epoxide hydrolase, microsomal epoxide hydrolase, and glutathione S-transferase activities. *Toxicol Appl Pharmacol.* 1983;71:254–265.
- Davis BB, Thompson DA, Howard LL, Morisseau C, Hammock BD, Weiss RH. Inhibitors of soluble epoxide hydrolase attenuate vascular smooth muscle cell proliferation. *Proc Natl Acad Sci U S A.* 2002;99:2222–2227.
- Fang X, Moore SA, Stoll LL, Rich G, Kaduce TL, Weintraub NL, Spector AA. 14,15-Epoxyeicosatrienoic acid inhibits prostaglandin E₂ production in vascular smooth muscle cells. *Am J Physiol.* 1998;275:H2113–H2121.
- Morisseau C, Goodrow MH, Newman JW, Wheelock CE, Dowdy DL, Hammock BD. Structural refinement of inhibitors of urea-based soluble epoxide hydrolases. *Biochem Pharmacol.* 2002;63:1599–1608.
- Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ . *Proc Natl Acad Sci U S A.* 1997;94:4312–4317.
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{- $\Delta\Delta$ CT} method. *Methods.* 2001;25:402–408.
- Cronet P, Petersen JF, Folmer R, Blomberg N, Sjoblom K, Karlsson U, Lindstedt EL, Bamberg K. Structure of the PPAR α and - γ ligand binding domain in complex with AZ 242; ligand selectivity and agonist activation in the PPAR family. *Structure.* 2001;9:699–706.
- Fang X, Hu S, Watanabe T, Weintraub NL, Snyder GD, Yao J, Liu Y, Shyy JY, Hammock BD, Spector AA. Activation of peroxisome proliferator-activated receptor α by substituted urea-derived soluble epoxide hydrolase inhibitors. *J Pharmacol Exp Ther.* 2005;314:260–270.
- Schoonjans K, Watanabe M, Suzuki H, Mahfoudi A, Krey G, Wahli W, Grimaldi P, Staels B, Yamamoto T, Auwerx J. Induction of the acyl-coenzyme A synthetase gene by fibrates and fatty acids is mediated by a peroxisome proliferator response element in the C promoter. *J Biol Chem.* 1995;270:19269–19276.
- Mascaro C, Acosta E, Ortiz JA, Marrero PF, Hegardt FG, Haro D. Control of human muscle-type carnitine palmitoyltransferase I gene transcription by peroxisome proliferator-activated receptor. *J Biol Chem.* 1998;273:8560–8563.
- Varanasi U, Chu R, Huang Q, Castellon R, Yeldandi AV, Reddy JK. Identification of a peroxisome proliferator-responsive element upstream of the human peroxisomal fatty acyl coenzyme A oxidase gene. *J Biol Chem.* 1996;271:2147–2155.
- Mayes P, Botham K. Metabolism of unsaturated fatty acids and eicosanoids. In: Foltin J, Ransom J, Oransky J, eds. *Harper's Illustrated Biochemistry.* New York: McGraw Hill Co; 2003:232–240.
- Meade EA, McIntyre TM, Zimmerman GA, Prescott SM. Peroxisome proliferators enhance cyclooxygenase-2 expression in epithelial cells. *J Biol Chem.* 1999;274:8328–8334.
- Fischer JW, Hawkins S, Clowes AW. Pharmacologic inhibition of nitric oxide synthases and cyclooxygenases enhances intimal hyperplasia in balloon-injured rat carotid arteries. *J Vasc Surg.* 2004;40:115–122.
- Mitchell JA, Evans TW. Cyclooxygenase-2 as a therapeutic target. *Inflamm Res.* 1998;47(suppl 2):S88–S92.
- Seidler J, McGovern SL, Doman TN, Shoichet BK. Identification and prediction of promiscuous aggregating inhibitors among known drugs. *J Med Chem.* 2003;46:4477–4486.
- Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature.* 2000;407:538–541.
- Govind AP, Thampam RV. Proteins interacting with the mammalian estrogen receptor: proposal for an integrated model for estrogen receptor mediated regulation of transcription. *J Cell Biochem.* 2001;80:571–579.
- Brummer D, Berger JP, Liu J, Kintscher U, Wakino S, Fleck E, Moller DE, Law RE. A non-thiazolidinedione partial peroxisome proliferator-activated receptor γ ligand inhibits vascular smooth muscle cell growth. *Eur J Pharmacol.* 2003;466:225–234.
- Yu Z, Xu F, Huse LM, Morisseau C, Draper AJ, Newman JW, Parker C, Graham L, Engler MM, Hammock BD, Zeldin DC, Kroetz DL. Soluble epoxide hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids. *Circ Res.* 2000;87:992–998.
- Zhao X, Yamamoto T, Newman JW, Kim IH, Watanabe T, Hammock BD, Stewart J, Pollock JS, Pollock DM, Imig JD. Soluble epoxide hydrolase inhibition protects the kidney from hypertension-induced damage. *J Am Soc Nephrol.* 2004;15:1244–1253.
- Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, Liao JK. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. *Science.* 1999;285:1276–1279.
- Yang B, Graham L, Dikalov S, Mason RP, Falck JR, Liao JK, Zeldin DC. Overexpression of cytochrome P450 CYP2J2 protects against hypoxia-reoxygenation injury in cultured bovine aortic endothelial cells. *Mol Pharmacol.* 2001;60:310–320.
- Narravula S, Colgan SP. Hypoxia-inducible factor 1-mediated inhibition of peroxisome proliferator-activated receptor α expression during hypoxia. *J Immunol.* 2001;166:7543–7548.
- Yue TL, Bao W, Jucker BM, Gu JL, Romanic AM, Brown PJ, Cui J, Thudium DT, Boyce R, Burns-Kurtis CL, Mirabile RC, Aravindhan K, Ohlstein EH. Activation of peroxisome proliferator-activated receptor- α protects the heart from ischemia/reperfusion injury. *Circulation.* 2003;108:2393–2399.
- Seubert J, Yang B, Bradbury JA, Graves J, Degraff LM, Gabel S, Gooch R, Foley J, Newman J, Mao L, Rockman HA, Hammock BD, Murphy E, Zeldin DC. Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K⁺ channels and p42/p44 MAPK pathway. *Circ Res.* 2004;95:506–514.
- Harrington LS, Falck JR, Mitchell JA. Not so EEZE: the 'EDHF' antagonist 14, 15 epoxyeicosanoic acid has vasodilator properties in mesenteric arteries. *Eur J Pharmacol.* 2004;506:165–168.
- Gauthier KM, Deeter C, Krishna UM, Reddy YK, Bondiella M, Falck JR, Campbell WB. 14,15-Epoxyeicosanoic acid: a selective epoxyeicosatrienoic acid antagonist that inhibits endothelium-dependent hyperpolarization and relaxation in coronary arteries. *Circ Res.* 2002;90:1028–1036.